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BIOTECHNICAL METHODS SECTION (BTS)

Cloning of human thymic stromal lymphopoietin (TSLP) and signaling mechanisms leading to proliferation

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Thymic stromal lymphopoietin (TSLP) is a novel cytokine that was found to promote the development of murine B cells *in vitro*. Here we describe the cloning and characterization of the human homologue of murine TSLP. This protein, which is expressed in a number of tissues including heart, liver and prostate, prevented apoptosis and stimulated growth of the human acute myeloid leukemia (AML)-derived cell line MUTZ-3. Anti-interleukin (IL)-7 receptor antibodies (Abs) neutralized this effect indicating that TSLP binds to at least part of the IL-7 receptor complex. TSLP induced phosphorylation of signal transducer and activator of transcription (STAT)-5. In contrast to IL-7, TSLP-triggered STAT-5 phosphorylation was not preceded by activation of janus kinase (JAK) 3. These findings would be in accordance with the notion, raised previously for the mouse system, that TSLP leads to STAT-5 phosphorylation by activating other kinases than the JAKs. Some other signaling pathways stimulated by many cytokines are not involved in TSLP activity; thus, TSLP did not stimulate activation of ERK1,2 and p70S6K. Furthermore, neutralizing Abs raised against cytokines known to stimulate the growth of MUTZ-3 cells did not inhibit the proliferative effects of TSLP, suggesting that TSLP-induced growth was a direct effect. In summary, we describe the cloning of human TSLP and its proliferative effects on a myeloid cell line. TSLP-induced proliferation is preceded by phosphorylation of STAT-5, but not of JAK 3. *Leukemia* (2001) 15, 1286–1292.

Keywords: TSLP; signal transduction; cell lines; apoptosis

Introduction

A number of cytokines, such as interleukin (IL)-7, play a key role in the development of B cells. A recently cloned member of this family of B cell-stimulating factors is thymic stromal lymphopoietin (TSLP), which has many effects on B cells that resemble those of IL-7.[1] Both IL-7 and TSLP interact with the alpha chain of the IL-7 receptor (IL-7Rα).[2,3] However, IL-7 but not TSLP, also interacts with the common gamma (γ_c) chain (part of the receptor complex for IL-2, IL-4, IL-7, IL-9 and IL-15). A second component of TSLP signaling is a unique receptor chain that specifically binds TSLP and is a member of the hematopoietin receptor superfamily.[3–6] TSLP binds to its unique receptor with low affinity; both the IL-7Rα chain as well as the unique TSLP receptor (TSLP R) chain are required for high affinity binding of TSLP to cells.[3] Signaling through the TSLP R is unique in that it triggers tyrosine phosphorylation of signal transducer and activator of transcription (STAT)-5 without activating any of the known janus kinases (JAK).[2]

All studies of the biological activities of murine TSLP have been performed on murine cells, since this protein appears to be ineffective on human hematopoietic cells.[3,7] We now describe the cloning and characterization of what we believe to be the human homologue of murine TSLP. The human homologue is 43% identical at the amino acid level to the mouse protein. In our search for a human TSLP-responsive model system, we tested a panel of cytokine-dependent human hematopoietic cell lines. These cells are widely used in cytokine bioassays, as they die without the appropriate growth factors and allow for the quantitation of the respective factor.[8] We show that the acute myeloid leukemia (AML)-derived cell line MUTZ-3 represents a suitable bioassay for human TSLP, which induces the growth and prevents the apoptosis of these cells. Furthermore, TSLP induces activation of STAT-5, but not of JAK 3, confirming that an unknown kinase might be responsible for STAT-5 phosphorylation.

Material and methods

Cell culture

The continuous cell lines were either taken from the stock of the cell bank (DSMZ, German Collection of Microorganisms and Cell Cultures)[9] or were generously provided by the original investigators. F-36P (AML M6),[10] HU-3 (AML M7),[11] M-07e (AML M7),[12] M-MOK (AML M7),[13] SKNO-1 (AML M2),[14] and TF-1 (AML M6)[15] cells were maintained in RPMI 1640 medium (Life Technologies, Karlsruhe, Germany) with 10% fetal bovine serum (FBS; Sigma, Deisenhofen, Germany) and 10% conditioned medium (CM) from the bladder carcinoma cell line 5637, which secretes several cytokines including granulocyte colony-stimulating factor (G-CSF) and granulocyte–macrophage CSF (GM-CSF).[16] MUTZ-3 (AML M4),[17] OCI-AML-1 (AML M4),[18] OCI-AML-5 (AML M4)[19] and UT-7 cells (AML M7)[20] were grown in α MEM medium (Life Technologies) with 10% FBS and 10% 5637 CM. The mouse pre-B cell line PB-1[21] was kept in McCoy's 5A with 15% FBS and 50 ng/ml hIL-7. All cell lines were free of contamination with mycoplasmas and were harvested in the logarithmic

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growth phase with a viability exceeding 85% as determined by trypan blue dye exclusion.

Growth factors, antibodies and inhibitors

The recombinant human (rh) versions of the various cytokines were employed, unless otherwise indicated. Tumor necrosis factor alpha (TNFα) was purchased from DPC Biermann (Bad Nauheim, Germany). GM-CSF, IL-3, rabbit anti-STAT-5B antiserum and neutralizing antibodies (Abs) against IL-3, IL-6, IL-7, G-CSF, GM-CSF, macrophage-CSF (M-CSF) and stem cell factor (SCF) were obtained from R&D Systems (Wiesbaden, Germany). Mouse anti-STAT-5 monoclonal Ab (mAb) was obtained from Dianova (Hamburg, Germany). Mouse anti-phosphotyrosine mAb 4G10 was purchased from Biomol/Upstate Biotechnology (Hamburg, Germany). Rabbit anti JAK 3 antiserum and mouse anti-pErk mAb were purchased from Santa Cruz (Heidelberg, Germany).

Cloning of human TSLP

A cDNA (AA889581) with 56% nucleotide sequence homology to murine TSLP was identified as an EST in a human testis cDNA library and cloned (IMAGE 1407260). The EST contained what appeared to be part of a sequence encoding human TSLP, but was shorter in length than the murine sequence and appeared to be missing amino acids at the C-terminal end. In order to isolate the remaining coding region, a variety of cDNA libraries were screened by polymerase chain reaction (PCR) using TSLP-specific primers for expression of this cDNA. Two independently derived cDNAs representing the 3′ end of human TSLP were cloned from a Clontech (Palo Alto, CA, USA) testes library, and each clone encoded the same sequence of human TSLP. The longer of these two sequences contained an additional 15 amino acids of coding region as well as 75 base pairs of 3′ untranslated region. PCR was used to join this sequence and the original AA889581 EST to create a full length TSLP cDNA. The full length TSLP gene was cloned into the expression vector pDC 409.[22]

Human TSLP protein purification

PCR was used to fuse the full length TSLP sequence to nucleic acid sequences encoding six histidine residues; this poly-histidine tag allows the protein to be purified on a nickel column. In addition to the histidine residues, eight amino acids (the FLAG sequence) were also added to the C-terminal end of the protein. The FLAG sequence allows the protein to be recognized by antibodies directed against the FLAG sequence.[23] This sequence with both tags on it (TSLP-HF) was cloned into the expression vector pDC 409, in which expression of the protein is driven by a CMV promoter. rh TSLP-HF protein was made by transfecting CV-1/EBNA cells with the plasmid containing the TSLP-HF protein, and then 3 days later purifying the protein over a Ni^{2+} column. Purity of the TSLP-HF protein was verified by staining of the protein following gel electrophoresis as well as by N-terminal sequencing.

Northern blot analysis

Human tissue blots I and II (Clontech) were probed for human TSLP mRNA expression. The following sequence was used as a primer for the generation of a radioactive antisense PCR probe, using the human TSLP cDNA as a template: 5′-TGTTGTTTCAGTAAAGGTCGATTGAAGCGA-3′. Blots were prehybridized 6 h at 55°C and then hybridized overnight with 2×10^6 cpm/ml at 55°C. Blots were washed in 2 × SSC, 0.1% SDS at 50°C several times and then washed once at 55°C for 1 h before being exposed to film for 72 h.

[^{3}H]-thymidine uptake and analysis of cell growth and viability

Assays of [^{3}H]-thymidine incorporation were done as follows: 2.5×10^4 cells (in 100 μl) were seeded in triplicate in 96-well flat-bottom microtiter plates after washing and resuspension in growth factor-free medium. Effectors were added as 2× concentrated solutions in a 100 μl volume or as 4× concentrated solutions in a 50 μl volume. For the last 3 h of the incubation period, 1 μCi [^{3}H]-thymidine (Amersham Pharmacia Biotech, Freiburg, Germany) was added to each well. Cells were harvested on glass fiber filters with a multiple automatic sample harvester, and radioactivity was determined in a liquid scintillation counter.

Detection of apoptotic cells

Apoptotic cells were detected and quantitated with the annexin-V/propidium iodide (PI) method, applying a commercially available kit (R&D). Cells were washed with medium without FBS and cytokines and resuspended in medium with 0.1% bovine serum albumin (BSA) with or without TSLP or other cytokines. Binding of fluorescein isothiocyanate (FITC)-labeled annexin-V and PI staining of the cells was determined after 19 h by flow cytometry analysis using a FACScan (Becton Dickinson, Heidelberg, Germany).

Immunoprecipitation and Western blot analysis

Analysis of STAT-5 and JAK 3 phosphorylation was done as follows: 2×10^7 cells/analysis were washed free of growth factor-containing medium and cultured overnight in factor-free and serum-free medium (0.1% BSA) prior to stimulation with TSLP (100 ng/ml in 1 ml), IL-3 (100 ng/ml in 1 ml), or IL-7 (100 ng/ml in 1 ml). After stimulation, the cells were pelleted and washed with ice-cold phosphate-buffered saline (PBS). For STAT-5 immunoprecipitation, cells were lysed with 1 ml NP40 lysis buffer (20 mM Tris-HCL pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% NP40, 0.02% NaN_3, 1 mM Na orthovanadate, 2 μg/ml aprotinin, 5 μg/ml leupeptin, 20 μM antipain, 20 μM pepsatin, 10 mM NaF, 0.2 mg/ml PMSF). For JAK 3 immunoprecipitation, the lysis buffer consisted of PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS with the above inhibitors. 3.5 μg anti-STAT-5B or anti JAK 3 Abs were added to the precleared lysates. After 1 h rotation (4°C) the immunoprecipitates were collected by adding protein G sepharose (45 min). The immunoprecipitates were washed twice with ice-cold lysis buffer, twice with 0.5 M LiCl, 100 mM Tris-HCl pH 8.0, and boiled for 10 min in 35 μl SDS sample buffer containing 15% glycerol, 125 mM Tris-HCL pH 6.8, 5 mM EDTA, 2%

SDS, 0.1% bromophenol blue and 1% β-mercaptoethanol. The samples (10 μl for STAT-5 or JAK 3 detection; 20 μl for phosphotyrosine detection) were subjected to SDS-gel electrophoresis on polyacrylamide gels using the Protean II chamber (BioRad, München, Germany). The samples were separated on a 9% gel and electroblotted on to nitrocellulose membranes (trans-blot transfer medium; BioRad). The membranes were labeled overnight with the respective Abs, and specific bands were visualized with the biotin/streptavidin-horseradish peroxidase system (Amersham) in combination with the Renaissance Western Blot Chemoluminescence Reagent protocol (Du Pont, NEN, Bad Homburg, Germany).

Results

Nucleotide and amino acid sequence of putative human TSLP

A cDNA clone (AA889581) with 56% nucleotide sequence homology to murine TSLP was identified in a human testis cDNA library during a search of the EST database for a human homologue of murine TSLP. The cDNA was purchased and used to clone a putative, full-length human homologue of the murine TSLP gene, as described in Materials and methods. The nucleotide sequence of the putative human TSLP gene is 740 bp long and encodes a protein of 159 amino acids (Figure 1). There are 196 bp of 5′ and 64 bp of 3′ sequence flanking the coding region. Cleavage of a signal peptide after the threonine residue at amino acid 28 is predicted using the GCG Signalpep program (score = 4.0), leaving a mature protein of 131 amino acids. Notably, there is a stretch of seven basic amino acids (KKRRKRK) near the C-terminal end of the protein. Radiation hybrid mapping was used to localize the putative human TSLP gene to chromosome 5, 3.87 cR from NIB916 and 8.56 cR from D5S492 (data not shown). This section of human chromosome 5 is syntenic with mouse chromosome 18, where the murine TSLP gene has previously been localized.[7] The mapping data support our hypothesis that the gene described in this paper is the true human homologue of murine TSLP.

Comparison of human and mouse TSLP amino acid sequences

A comparison of the putative human and murine TSLP amino acid sequences is shown in Figure 2. The two proteins are 43% identical at the amino acid level, as determined by the GCG GAP program. There are two potential sites for N-linked glycosylation in the mature human TSLP amino acid sequence (Figure 1). Note, however, that the position of residues representing potential sites for N-linked glycosylation are not conserved between the mouse and human proteins (Figure 2). The position of six of the seven cysteine residues present in the mouse TSLP sequence are conserved in the human TSLP sequence. Interestingly, the only cysteine residue not conserved from the mouse sequence is the second cysteine residue. Intramolecular disulfide bonding patterns of the cysteine residues in mouse TSLP show the following pairings: cysteine one bonds with six, three bonds with four, and five bonds with seven.[7] Thus, the only cysteine residue not involved in disulfide bond formation in murine TSLP is the non-conserved second cysteine residue, which is missing in the human TSLP protein.

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     GCAGCCAGAAAGCTCTGGAGCATCAGGGAGACTCCAACTTAAGGCAACAGCATGGGTG
   AATAAGGGCTTCCTGTGGACTGGCAATGAGAGGCAAAACCTGGTGCTTGAGCACTGGCCCC
   TAAGGCAGGCCTTACAGATCTCTTACACTCGTGGTGGGAAGAGTTTAGTGTGAAACTGGGG
   TGGAATTGGGTGTCCACGTATGTTCCCTTTTGCCTTACTATATGTTCTGTCAGTTTCTTTC
 1                   M  F  P  F  A  L  L  Y  V  L  S  V  S  F

   AGGAAAATCTTCATCTTACAACTTGTAGGGCTGGTGTTAACTTACGACTTCACTAACTGT
15   R  K  I  F  I  L  Q  L  V  G  L  V  L  T  Y  D  F  T  N  C

   GACTTTGAGAAGATTAAAGCAGCCTATCTCAGTACTATTTCTAAAGACCTGATTACATAT
35   D  F  E  K  I  K  A  A  Y  L  S  T  I  S  K  D  L  I  T  Y

   ATGAGTGGGACCAAAAGTACCGAGTTCAACAACACCGTCTCTTGTAGCAATCGGCCACAT
55   M  S  G  T  K  S  T  E  F  N  N  T  V  S  C  S  N  R  P  H

   TGCCTTACTGAAATCCAGAGCCTAACCTTCAATCCCACCGCCGGCTGCGCGTCGCTCGCC
75   C  L  T  E  I  Q  S  L  T  F  N  P  T  A  G  C  A  S  L  A

   AAAGAAATGTTCGCCATGAAAACTAAGGCTGCCTTAGCTATCTGGTGCCCAGGCTATTCG
95   K  E  M  F  A  M  K  T  K  A  A  L  A  I  W  C  P  G  Y  S

   GAAACTCAGATAAATGCTACTCAGGCAATGAAGAAGAGGAGAAAAAGGAAAGTCACAACC
115  E  T  Q  I  N  A  T  Q  A  M  K  K  R  R  K  R  K  V  T  T

   AATAAATGTCTGGAACAAGTGTCACAATTACAAGGATTGTGGCGTCGCTTCAATCGACCT
135  N  K  C  L  E  Q  V  S  Q  L  Q  G  L  W  R  R  F  N  R  P

   TTACTGAAACAACAGTAAACCATCTTTATTATGGTCATATTTCACAGCCCAAAATAAATC
155  L  L  K  Q  Q  *

   ATCTTTATTAAGTAAAAAAAAA
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Figure 1 Nucleotide and amino acid sequence of human TSLP. The putative signal sequence is underlined, and the two potential sites of N-linked glycosylation are boxed. The start codon (ATG) and stop codon (TAA) are shown in bold type.

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Human      1 MFPFALLYVLSVSFRKIFILQ.LVGLVLTYDFTNCDFEKIKAAYLSTISK 49
                 |  | :|||| || : |||.|.||.|  |   | . |
Mouse      1          MVLLRSLFILQVLVRMGLTYNFSNCNFTSITKIYCNIIFH 40

Human     50 DLITYMSGTKSTEFNNTVSCSNRPHCLTEIQSLTFNPTAGCASLAKEMFA 99
             ||   : | |   |     | .:| || .|:  | ||  || ||  . ||
Mouse     41 DLTGDLKGAK...FEQIEDCESKPACLLKIEYYTLNPIPGCPSLPDKTFA 87

Human    100 MKTKAALAIWCPGYSETQIN.ATQAMKKRRKRKVTTNKCLEQVSQLQGLW 148
              :|: ||   |||| ||: |  || | .        | ||  | ||:  ||
Mouse     88 RRTREALNDHCPGYPETERNDGTQEMAQE.....VQNICLNQTSQILRLW 132

Human    149 RRFNRPLLKQQ 159
                |  .
Mouse    133 YSFMQSPE   140
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Figure 2 Comparison of human and mouse TSLP amino acid sequences. The putative signal sequences are underlined, potential sites of N-linked glycosylation are boxed, and cysteine residues are highlighted in bold type.

Northern blot analysis showing TSLP expression in human tissue

The mRNA representing the putative human TSLP transcript is expressed in many tissues, as determined by Northern blot analysis (Figure 3). The size of the TSLP mRNA is approximately 1.0–1.2 kb in almost every tissue, but larger size mRNAs are seen in the testis. The mRNA size is consistent with the overall length (1000 bp) of the cDNA clone isolated (Figure 1). Highest expression levels were seen in heart, liver, testis and prostate, with lower expression in lung, skeletal muscle, kidney, spleen, ovary, small intestine and colon. Human TSLP mRNA appears by Northern blot analysis to have a more widespread tissue distribution pattern than was observed with murine TSLP mRNA.[7]

TSLP induces cell growth of AML-derived cell line MUTZ-3

Human TSLP was tested on 10 cytokine-dependent human myeloid cell lines in order to find a bioassay for this novel cytokine. A positive or negative influence of TSLP on the proliferation of the cells was tested by assaying the rate of [^{3}H]-thymidine incorporation, using TSLP alone or in combination with other cytokines. Only MUTZ-3 cells, but not F-36P, HU-3, M-07e, M-MOK, OCI-AML-1, OCI-AML-5, SKNO-1, TF-1, or UT-7 cells were responsive to TSLP (data not shown). The responsiveness of MUTZ-3 cells was demonstrated in dose-response assays (Figure 4), as well as in time-course studies (Figure 5). There is, however, a certain variability in responsiveness to TSLP with with S.I. between 1.7 and 6.5 for 10 ng/ml TSLP in different experiments. GM-CSF, IL-3, and various other growth factors showed additive proliferative effects in combination with TSLP. In contrast, TSLP could not counteract the apoptosis-inducing activity of TNFα (Figure 6).

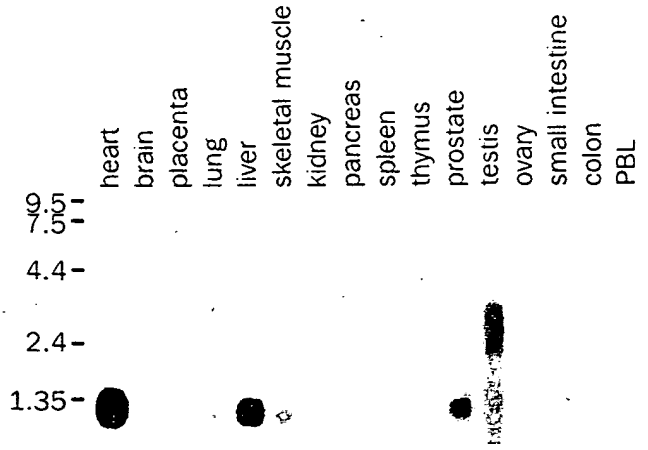

Figure 3 Northern blot analysis showing TSLP expression in human tissues. Human tissue blots were probed as described in Material and methods.

TSLP inhibits apoptosis

MUTZ-3 cells are consistently and absolutely cytokine-dependent. Removal of cytokines causes the cells to stop proliferation and eventually to die. An early indicator of apoptosis is the induced capacity of cells to bind annexin-V. Annexin-V binds to phosphatidylserine residues that are found on the inner leaflet of the cell membrane, but that translocate to the outer leaflet during apoptosis. In contrast, PI enters the cell later during apoptosis after the loss of cell membrane integrity. In MUTZ-3 cells, removal of growth factors clearly induced apoptosis (Table 1). TSLP significantly reduced annexin-V and PI staining in these cells, demonstrating its capacity to replace other cytokines as an antiapoptotic factor (Table 1).

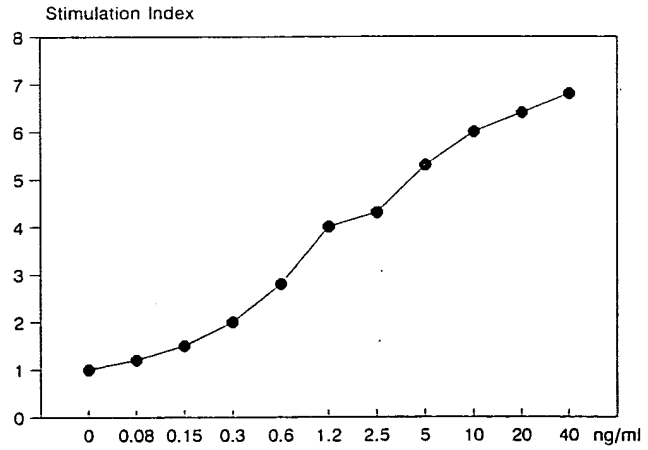

Figure 4 Dose-response curve of MUTZ-3 cells incubated with TSLP. The cells were cultured with different concentrations of TSLP for 72 h. Cellular proliferation was measured by the standard [^{3}H]-thymidine assay. Results are expressed as stimulation index relative to the untreated control cells (SI = 1).

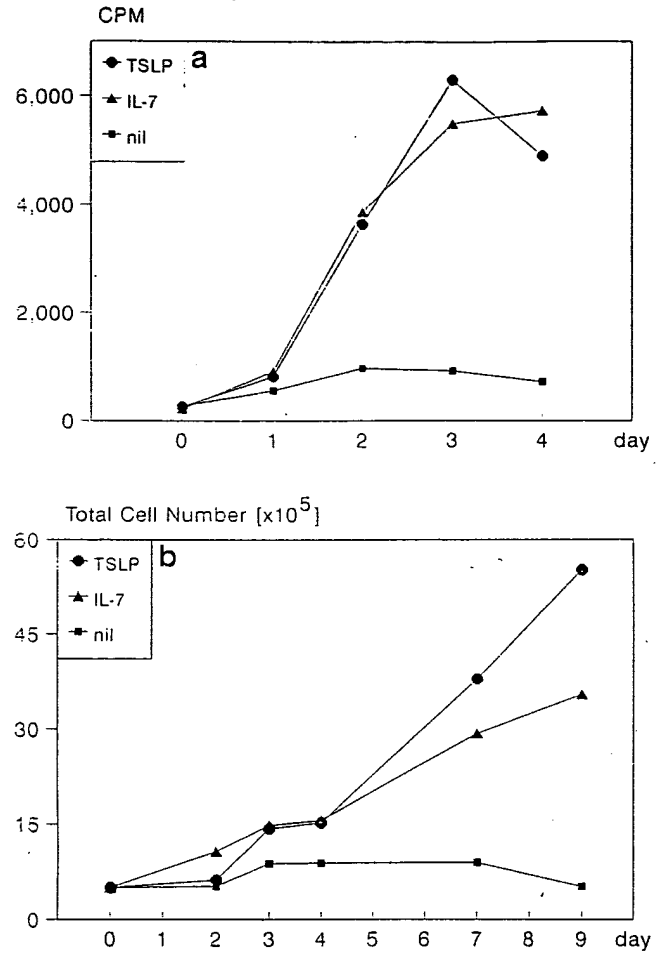

Figure 5 Time-course curves of the proliferative effects of TSLP and IL-7 on MUTZ-3 cells. Cells were cultured with TSLP (10 ng/ml) or IL-7 (10 ng/ml). (a) [^{3}H]-thymidine incorporation was determined after the times indicated. (b) Cell numbers were counted after the times indicated.

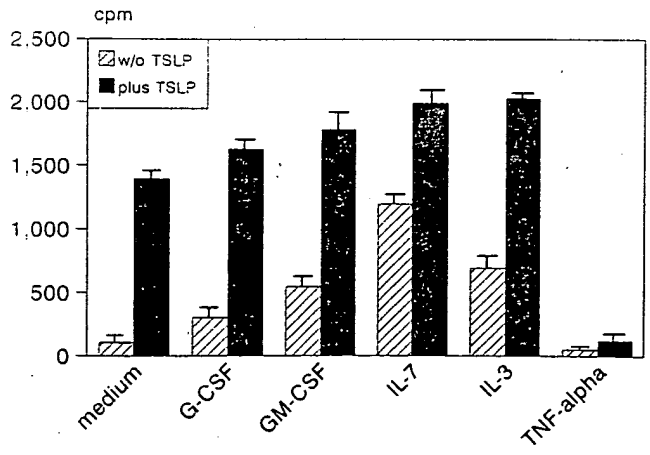

Figure 6 Additive or inhibitory effects of various cytokines on TSLP-induced proliferation. MUTZ-3 cells were cultured for 3 days with TSLP (10 ng/ml) and various cytokines: IL-6 (10 ng/ml), G-CSF (10 ng/ml), GM-CSF (0.1 ng/ml), IL-7 (10 ng/ml), IL-3 (0.1 ng/ml), TNFα (10 ng/ml). Cellular proliferation was measured by the standard [^{3}H]-thymidine assay. Apoptosis induced by TNFα was assessed by annexin-V staining.

Table 1 Antiapoptotic response of MUTZ-3 cells to TSLP

	Annexin V$^-$/PI$^-$	*Annexin-V$^+$/PI$^-$*	*Annexin-V$^+$/PI$^+$*
nil	23%	25%	52%
TSLP	52%	17%	31%
IL-7	42%	19%	39%
GM-CSF	77%	12%	11%

Cells were kept for 19 h in medium with or without growth factors and stained with FITC-conjugated annexin-V and propidium iodide (PI). Data are expressed as percent positive cells, determined by flow cytometry analysis. Viable cells are annexin V$^-$/PI$^-$; annexin-V$^+$/PI$^-$ cells are early apoptotic, annexin-V$^+$/PI$^+$ late apoptotic. Shown are data from one representative experiment.

Anti-IL-7R Abs inhibit TSLP-induced proliferation

It had been shown previously that proliferation of mouse pre-B cells in response to murine TSLP was inhibited by anti-IL7Rα Abs. These data led to the hypothesis that TSLP conveyed its effects by binding to the IL-7R.[2] A first indication that this also occurs in human cells was our observation that MUTZ-3, but none of the TSLP-unresponsive cell lines, proliferated upon stimulation with IL-7.[8] Thus, the cell line definitely expressing the IL-7R responded to TSLP, while the IL-7 unresponsive cells were TSLP-unresponsive. That human TSLP may indeed bind to the human IL-7Rα chain was supported, though not definitely proven, by the findings that anti-IL-7R Abs inhibited TSLP-induced proliferation (Figure 7). The Ab concentration necessary to inhibit the effects of TSLP (20 μg/ml), however, was 10-fold higher than the one that was required to inhibit IL-7. This suggests that TSLP might bind in a different mode to the IL-7R than IL-7 itself. These data would be consistent with the idea that there is a separate receptor for human TSLP that is complexed with the human IL-7Rα chain.[3]

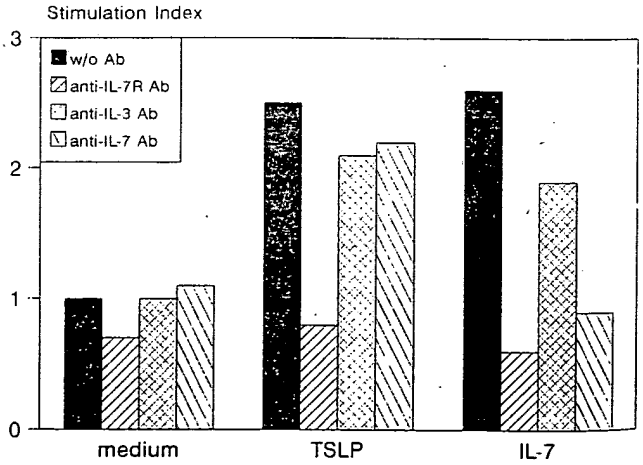

Figure 7 Effects of inhibitory Abs on TSLP-induced proliferation. MUTZ-3 cells were incubated for 3 days with TSLP (2 ng/ml) or IL-7 (2 ng/ml) and with neutralizing Abs raised against IL-7Rα (20 μg/ml) or IL-7 (2 μg/ml). An anti IL-3 mAb, applied in the same concentration as the anti-IL-7Rα mAb, confirmed that the high Ig concentration itself did not have toxic effects. Also, anti-IL-7Rα mAb did not or only marginally reduce IL-3-induced proliferation. Cellular proliferation was measured by the [^{3}H]-thymidine assay. Results are expressed as stimulation index relative to the untreated control cells (SI = 1) and show one representative experiment.

TSLP-induced proliferation is not mediated by induction of other growth factors

We had shown in a previous study that MUTZ-3 cells grow in response to IL-3, IL-6, IL-7, G-CSF, GM-CSF, M-CSF and SCF.[8] Recently, we have demonstrated that cytokines stimulate proliferation in some myeloid cell lines indirectly by inducing the production of one of the growth factors mentioned above.[24] Therefore, we used a panel of neutralizing Abs to test whether this was also the case in TSLP-induced growth. However, the proliferative effect of TSLP was not inhibited by neutralizing Abs raised against any of the above cytokines (data not shown). It appears that TSLP activates proliferation directly, and not by induction of another growth factor, at least not one of those examined here.

TSLP stimulates phosphorylation of STAT-5 without activation of JAK 3

In contrast to IL-3 or GM-CSF, TSLP did not induce tyrosine phosphorylation of ERK 1,2 or p70S6K in MUTZ-3 cells (data not shown). Thus, neither the MAPK pathway nor the p70S6K pathway appear to be involved in the signal transduction pathway elicited by TSLP. However, both IL-7 and TSLP induced tyrosine phosphorylation of STAT-5 (Figure 8). In contrast to IL-7, TSLP did not activate JAK 3 (Figure 8). For the mouse system, it has been shown that none of the JAKs are responsible for STAT phosphorylation suggesting that TSLP may activate some unknown STAT phosphorylating kinases.[2] Future studies will show whether this is also the case for human cells.

Discussion

TSLP was originally purified as a B cell stimulatory factor from the CM of murine thymic stromal cells.[1] It supports the proliferation and differentiation of committed murine B220+ B cell progenitors.[25] The effects of TSLP are similar to those of IL-7, not only in B cell lymphopoiesis, but also in their ability to support the growth of the mouse pre-B cell line NAG8/7 and to costimulate mouse thymocytes.[1] A major difference between the effects of TSLP and those of IL-7 is that TSLP promotes the development of B220+/IgM+ immature B cells, while IL-7 enhances the development of B220+/IgM– immature pre-B cells from fetal liver.[1] The similarities of the responses to IL-7 and TSLP might be explained in part by the findings that both cytokines bind to the IL-7Rα chain.[2,3] In contrast to the IL-7R, a specific TSLP R chain and not the γ_c chain completes the mouse TSLP R complex.[2,3,26] An understanding of the function of TSLP in the human hematopoietic system should now be possible with our cloning of the gene encoding the human TSLP protein.

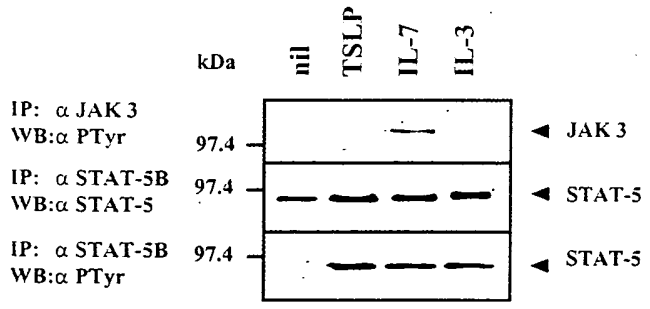

Figure 8 Influence of TSLP on the phosphorylation status of JAK 3 and STAT-5. JAK 3 and STAT-5 molecules were immunoprecipitated with the respective Abs (IP: αJAK 3 or αSTAT-5B). Proteins were separated by 9% SDS-PAGE and transferred to nitrocellulose membranes. Immunoblots were probed with the respective anti-phosphotyrosine mAb (WB: αPtyr), or anti STAT-5 mAb (WB: αSTAT-5). Short-term stimulation (25 min) with TSLP (100 ng/ml) induced tyrosine phosphorylation of STAT-5, but not of JAK 3. IL-7 (100 ng/ml) triggered stimulation of JAK 3 and STAT-5.

In our search for a bioassay for human TSLP, we screened a panel of human growth factor-dependent cell lines. These cells die after removal of cytokines, thus allowing for a quantitative detection of the respective growth factor. Most human cytokine-dependent cell lines are derived from AML and basically none from the B cell lineage are available. We speculated that the use of myeloid cells would not be a major obstacle as commonly the effects of IL-7 and of most other cytokines are not restricted to a specific cell lineage. IL-7, for example, has been shown to affect not only B cells, but induces also T cell proliferation and development, as well as cytokine synthesis in monocytes.[27–29]

MUTZ-3 was the only of the 10 cell lines tested that responded proliferatively to TSLP. The suitability of this cell line as a bioassay for human TSLP was shown both in dose-response and time-course studies. Most of the growth factors known to be active on MUTZ-3[8] showed an additive proliferative effect in combination with TSLP. The antiproliferative/apoptotic effect of TNFα, however, was dominant even in the presence of TSLP.

Interestingly, MUTZ-3 was not just the only cell line examined that proliferated after stimulation with TSLP, but also the only one that was IL-7 responsive. As indicated above, it had been shown in the mouse system, that TSLP binds to the IL-7Rα chain.[3] The effective neutralization of TSLP-induced proliferation of MUTZ-3 cells by anti-IL7Rα chain Abs clearly supports this notion. However, TSLP inhibition required a 10-fold higher Ab concentration than the inhibition of a comparable IL-7 effect, suggesting that the affinity and mode of binding to the receptor may be different between the two molecules.

Neither TSLP nor IL-7 activated the MAPK and p70S6K pathways. This is noteworthy as many cytokines binding to receptors of the cytokine receptor superfamily, such as IL-3 or GM-CSF, do stimulate these signaling chains.

Studies using neutralizing Abs have shown that TSLP signaling requires binding to the IL-7Rα chain, but – and this is in contrast to IL-7 signaling – not to the γ_c-chain.[2,3] JAK 3 is known to bind to the γ_c-chain.[30,31] Accordingly IL-7, but not TSLP induced activation of JAK 3 in mouse cells.[2] We found the same response pattern for the human system: TSLP induced STAT-5 tyrosine phosphorylation but, in contrast to IL-7, no JAK 3 stimulation. Future studies will show whether human TSLP fails to activate JAK family members in human cells just as murine TSLP fails to activate JAK proteins in mouse cells.[2] In this context it may be interesting that there are indications that Tec protein kinases might be responsible for STAT-5 phosphorylation.[26]

To date there have been four separate published reports of the cloning of the murine TSLP R,[3–6] although in two of these cases[4,5] the protein was cloned as an orphan receptor without the knowledge that it binds TSLP. Since the actual ligand binding to this receptor had remained unknown, signaling studies were performed using chimeric receptor constructs. The murine receptor did not stimulate JAK 1 or JAK 3,[5] consistent with our observation that stimulation of human TSLP R did not lead to JAK 3 phosphorylation.

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References

1 Friend SL, Hosier S, Nelson A, Foxworthe D, Williams DE, Farr AG. A thymic stromal cell line supports *in vitro* development of surface IgM+ B cells and produces a novel growth factor affecting B and T lineage cells. *Exp Hematol* 1994; **22**: 321–328.
2 Levin SD, Koelling RM, Friend SL, Isaksen DE, Ziegler SF, Perlmutter RM, Farr AG. Thymic stromal lymphopoietin: a cytokine that promotes the development of IgM+ B cells *in vitro* and signals via a novel mechanism. *J Immunol* 1999; **162**: 677–683.
3 Park LS, Martin U, Garka K, Gliniak B, DiSanto JP, Muller W, Largaespada DA, Copeland NG, Jenkins NA, Farr AG, Ziegler SF, Morrissey PR, Paxton R, Sims J. Cloning of the murine thymic stromal lymphophoietin (TSLP) receptor. Formation of a functional heteromeric complex requires IL-7 receptor. *J Exp Med* 2000; **192**: 659–670.
4 Hiroyama T, Iwama A, Morita Y, Nakamura Y, Shibuya A, Nakauchi H. Molecular cloning and characterization of CRLM-2, a novel type I cytokine receptor preferentially expressed in hematopoietic cells. *Biochem Biophys Res Comm* 2000; **272**: 224–229.
5 Fujio K, Nosaka T, Kojima T, Kawashima T, Yahata T, Copeland NG, Gilbert DJ, Jenkins NA, Yamamoto K, Nishimura T, Kitamura T. Molecular cloning of a novel type I cytokine receptor similar to the common gamma chain. *Blood* 2000; **95**: 2204–2211.
6 Pandey A, Ozaki K, Baumann H, Levin SD, Puel A, Farr AG, Ziegler SF, Leonhard WJ, Lodish HF. Cloning of a receptor subunit required for signaling by thymic stromal lymphopoietin. *Nature Immunol* 2000; **1**: 59–64.
7 Sims JE, Williams DE, Morissey PJ, Garka K, Foxworthe D, Price V, Friend S, Farr A, Bedell MA, Jenkins NA, Copeland NG, Grabstein K, Paxton RJ. Molecular cloning and biological characterization of a novel murine lymphoid growth factor. *J Exp Med* 2000; **192**: 671–680.
8 Drexler HG, Zaborski M, Quentmeier H. Cytokine response profiles of human myeloid factor-dependent leukemia cell lines. *Leukemia* 1997; **11**: 701–708.
9 Drexler HG, Dirks W, MacLeod RAF, Quentmeier H, Steube KG, Uphoff CC. *DSMZ Catalogue of Human and Animal Cell Lines*, 8th edn. DSMZ: Braunschweig, Germany, 2001.
10 Chiba S, Takaku F, Tange T, Shibuya K, Misawa C, Sasaki K, Miyagawa K, Yazaki Y, Hirai H. Establishment and erythroid differentiation of a cytokine-dependent human leukemic cell line F-36: a parental line requiring granulocyte–macrophage colony-stimulating factor or interleukin-3, and a subline requiring erythropoietin. *Blood* 1991; **78**: 2261–2268.
11 Morgan DA, Class R, Soslau G, Brodsky I. Cytokine-mediated erythroid maturation in megakaryoblastic human cell line HU-3. *Exp Hematol* 1997; **25**: 1378–1385.
12 Avanzi GC, Lista P, Giovinazzo, B, Miniero R, Saglio G, Benetton G, Coda R, Cattoretti G, Pegoraro L. Selective growth response to IL-3 of a human leukaemic cell line with megakaryoblastic features. *Br J Haematol* 1988; **69**: 359–366.
13 Itano M, Tsuchiya S, Minegishi N, Fujie H, Minegishi M, Morita S, Yambe T, Ohashi Y, Masuda T, Koike T, Konno T. Establishment and characterization of a novel human immature megakaryoblastic leukemia cell line, M-MOK, dependent on fibroblasts for its viability. *Exp Hematol* 1995; **23**: 1301–1309.
14 Matozaki S, Nakagawa T, Kawaguchi R, Aozaki R, Tsutsumi M, Murayama T, Koizumi T, Isobe RNT, Chihara K. Establishment of a myeloid leukaemic cell line (SKNO-1) from a patient with t(8;21) who acquired monosomy 17 during disease progression. *Br J Haematol* 1995; **89**: 805–811.
15 Kitamura T, Tange T, Terasawa T, Chiba S, Kuwaki T, Miayagawa K, Pia YF, Miazono K, Urabe A, Takaku F. Establishment and characterization of a unique human cell line that proliferates dependently on GM-CSF, IL-3, or erythropoietin. *J Cell Physiol* 1989; **140**: 323–334.
16 Quentmeier H, Zaborski M, Drexler HG. The human bladder carcinoma cell line 5637 constitutively secretes functional cytokines. *Leukemia Res* 1997; **21**: 343–350.
17 Hu ZB, Ma W, Zaborski M, MacLeod RAF, Quentmeier H, Drexler HG. Establishment and characterization of two novel cytokine-responsive acute myeloid and monocytic leukemia cell lines, MUTZ-2 and MUTZ-3. *Leukemia* 1996; **10**: 1025–1040.
18 Wang C, Curtis JE, Minden MD, McCulloch EA. Expression of a retinoic acid receptor gene in myeloid leukemia cells. *Leukemia* 1989; **3**: 264–269.
19 Wang C, Koistinen P, Yang GS, Williams DE, Lyman SD, Minden MD, McCulloch EA. Mast cell growth factor, a ligand for the receptor encoded by c-kit, affects the growth in culture of the blast cells of acute myeloblastic leukemia. *Leukemia* 1991; **5**: 493–499.
20 Komatsu N, Nakauchi H, Miwa A, Ishihara T, Eguchi M, Moroi M, Okada M, Sato Y, Wada H, Yawata Y, Suda T, Miura Y. Establishment and characterization of a human leukemic cell line with megakaryocytic features: dependency on granulocyte–macrophage colony-stimulating factor, interleukin 3, or erythropoietin for growth and survival. *Cancer Res* 1991; **51**: 341–348.
21 Mire-Sluis AR, Healey L, Griffiths S, Hockley D, Thorpe R. Development of a continuous IL-7-dependent murine pre-B cell line PB-1 suitable for the biological characterisation and assay of human IL-7. *J Immunol Meth* 2000; **236**: 71–76.
22 Giri JG, Eisenman AM, Shanebeck K *et al*. Utilization of the beta and gamma chains of the IL-2 receptor by the novel cytokine IL-15. *EMBO J* 1994; **13**: 2822–2830.
23 Hopp TP, Prickett KS, Price VL, Libby RT, March CJ, Cerretti DP. A short polypeptide marker useful for recombinant protein identification and purification: *Biotechnology* 1988; **6**: 1204–1210.
24 Quentmeier H, Dirks WG, Fleckenstein D, Zaborski M, Drexler HG. Tumor necrosis factor alpha-induced proliferation requires synthesis of granulocyte–macrophage colony-stimulating factor. *Exp Hematol* 2000; **28**: 1008–1015.
25 Ray RJ, Furlonger C, Williams DE, Paige CJ. Characterization of thymic stromal-derived lymphopoietin (TSLP) in murine B cell development *in vitro*. *Eur J Immunol* 1996; **26**: 10–16.
26 Isaksen DE, Baumann H, Trobridge PA, Farr AG, Levin SD, Ziegler SF. Requirement for Stat5 in thymic stromal lymphopoietin-mediated signal transduction. *J Immunol* 1999; **163**: 5971–5977.
27 Armitage RJ, Namen AE, Sassenfeld HM, Grabstein KH. Regulation of human T cell proliferation by IL-7. *J Immunol* 1990; **144**: 938–941.
28 Jicha DL, Mulé JJ, Rosenberg SA. Interleukin 7 generates antitumor cytotoxic T lymphocytes against murine sarcomas with efficacy in cellular adoptive immunotherapy. *J Exp Med* 1991; **174**: 1511–1515.
29 Alderson MR, Tough TW, Ziegler SF, Grabstein KH. Interleukin 7 induces cytokine secretion and tumoricidal activity by human peripheral blood monocytes. *J Exp Med* 1991; **173**: 923–930.
30 Miyazaki T, Kawahara A, Fuji H, Nakagawa Y, Minami Y, Liu ZJ, Oishi I, Silvennoinen O, Witthuhn BA, Ihle JN, Taniguchi T. Functional activation of Jak1 and Jak3 by selective association with IL-2 receptor subunits. *Science* 1994; **266**: 1045–1047.
31 Russel SM, Johnston JA, Noguchi M, Kawamura M, Bacon CM, Friedmann M, Berg M, McVicar DW, Witthuhn BA, Silvennoinen O, Goldman AS Schmalstieg FC, Ihle JN, O'Shea JJ, Leonard WJ. Interaction of IL-2R β and γ_c chains with Jak1 and Jak3: implications for XSCID and XCID. *Science* 1994; **266**: 1042–1045.